Synthesis of the Cholesteryl Ester Prodrugs Cholesteryl Ibuprofen and Cholesteryl Flufenamate and Their Formulation into Phospholipid Microemulsions

JOHN L. MURTHA[x] AND HOWARD Y. ANDO

Received May 10, 1993, from the *Philadelphia College of Pharmacy and Science, Department of Pharmaceutics, 600 S. 43rd Street, Philadelphia, PA 19104.* Accepted for publication February 28, 1994[⊗]. [x]Current address: Parke-Davis Pharmaceutical Research Division of Warner-Lambert Company, Department of Pharmaceutics, 2800 Plymouth Road, Ann Arbor, MI 48105.

Abstract □ Phospholipid microemulsions have been suggested as a drug-delivery system for hydrophobic compounds. In this study hydrophobicity was achieved by derivatizing with cholesterol. Cholesteryl ibuprofen (**3**) and cholesteryl flufenamate (**4**) were synthesized. **3** was isolated as an amorphous, white solid with a melting range of 114–120 °C. **4** was isolated as a crystalline, white solid with a melting range of 145–148 °C. The proposed structures of **3** and **4** were supported by IR, NMR, MS, and organic microanalysis. Phospholipid:cholesteryl ester microemulsions were prepared by the addition of a 1-propanol solution of the cholesteryl ester, other lipids, and phospholipid to a rapidly mixing KCl/KBr solution. The hydrophobic phase was modified by the addition of cholesteryl oleate or triolein to study the effect of the fluidity of the hydrophobic core on the formation of the microemulsions. The results indicated that a molar ratio of 75:25 and a total lipid concentration of 60 mg/mL consistently gave microemulsions with a mean size of 100–150 nm. In addition, the formation of eutectic mixtures of **3** and **4** with cholesteryl oleate were determined to be 16% (w/w) for **3** and 12% (w/w) for **4**; melting points were 35.2 and 45.2 °C, respectively. The solubilities of **3** and **4** in triolein were determined to be 13.2% (w/w) and 11.5% (w/w), respectively. Other investigators have shown that if the core of a phospholipid:cholesteryl estermicroemulsion exists in a liquid state at physiologic temperature, the turnover of the cholesteryl esters from these microemulsions occurs at a faster rate. Future studies will focus on the turnover of cholesteryl ester prodrug fluidized cores on the bioavailability of the free drug in vivo.

I. Introduction

Lipoproteins and microemulsions have distinct advantages for use as drug-delivery systems over liposomes or vesicles.[1] They have in plasma a higher physical stability than vesicles, their hydrophobic internal phase is resistant to leaching, and hydrophobic drugs can be solubilized in the internal phase. In this study, two acidic drugs, ibuprofen and flufenamic acid, were made hydrophobic by forming their cholesteryl ester derivatives.

Lipid microemulsions have been defined to have a droplet size ranging from 10–250 nm[1–4] and exist in a state between a micellar dispersion and a microemulsion.[1] Several methods have been described for the preparation of lipid microemulsions; ultrasonication[5–7] is the most common. Although this method is capable of producing 20–100-nm microemulsions, long sonication times (1–12 h) can potentially lead to lipid and drug degradation. For this study, a milder technique, which has also been used to prepare liposomes, has been chosen. Cholesteryl ester and phospholipid are dissolved in 1-propanol and then added to the aqueous phase with vigorous stirring.[8,9] Formulation variables that could have an effect on microemulsion particle size and bioavailability of the cholesteryl ester prodrugs was also investigated.

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1994.

II. Experimental Section

Reagents—Most reagents were used as received from the supplier: acetonitrile (HPLC grade), chloroform, cholesterol, hexane, methanol (HPLC grade), potassium chloride, potassium bromide, 1-propanol, 2-propanol, sodium phosphate dibasic, and tetrahydrofuran (HPLC grade) (Fisher Scientific Co., Pittsburgh, PA); cholesteryl oleate, L-α-dipalmitoylphosphatidylcholine, cholesteryl ester hydrolase (from porcine pancreas), cholesterol oxidase, flufenamic acid, horseradish peroxidase, *p*-hydroxyphenylacetic acid, 4-pyrrolidinopyridine, and triolein (Sigma Chemical Co., St. Louid, MO); 1,3-dicyclohexylcarbodiimide, polyethylene glycol 8000, and sodium taurocholate (Aldrich Chemical Co., Milwaukee, WI); ibuprofen (Mallinckrodt, St. Louis, MO; as a racemic mixture of 43% *R* and 53% *S* as determined by HPLC using the method of Berthod et al.[10]); ethyl acetate (EM Science, Cherry Hill, NJ); and 0.9% sodium chloride for injection USP (McGaw Inc., Irvine, CA). Anhydrous methylene chloride (Fisher Scientific, Pittsburgh, PA) was prepared by distillation over calcium hydride (Matheson Coleman & Bell, East Rutherford, NJ) and used immediately.[11]

Column Chromatography—The cholesteryl ester prodrugs were purified using column chromatography.[12,13] Silica gel (0–63 μm, Universal Scientific, Atlanta, GA) columns (2.5 cm diameter × 7 cm length) were prepared and equilibrated with hexane. A hexane solution of the reaction products was applied to the column and eluted with hexane. Approximately 10-mL fractions were collected using a fraction collector (FOXY 200 X-Y fraction collector, Isco, Inc., Lincoln, NE). The column effluent was monitored with a UV detector (SpectroMonitor III, Laboratory Data Control, Div. of Milton Roy Co., River Beach, FL) at 254 nm with the detector output recorded on a chart recorder (Perkin-Elmer Corp., Norwalk, CT).

Thin-Layer Chromatography—The synthetic reaction was monitored by TLC on silica gel plates (Whatman, silica gel, 60 Å, 1 × 3 in. 250-μm layer). Plates were developed using 80:20 hexane:ethyl acetate (R_f values: DCC = 0.02, cholesterol = 0.21, **1** = 0.56, **2** = 0.28, **3** = 0.77, **4** = 0.78).

Eluent fractions from column chromatography were monitored with TLC on silica gel plates (Fisher Scientific, Silica Gel GF, 20 cm × 20 cm, 250-μm layer). Plates were scored to form 20 1-cm-wide channels. Plates were developed using 80:20 hexane:ethyl acetate (R_f values: **3** = 0.75, **4** = 0.76) to verify only one compound was present in each fraction.

Melting Points—Two methods were used to determine melting ranges: a capillary melting point apparatus (A. H. Thomas Co., Philadelphia, PA) and a differential scanning calorimeter (DSC) (TA 3000 System with TC10 TA processor and DSC20 standard cell, Mettler Instrument Corp., Hightstown, NJ). Samples of 10–15 mg were weighed into vented DSC pans and scans run from 25 to 200 °C at 5 °C/min.

Infrared Spectroscopy—Infrared spectra of the reaction products were obtained on a FT-IR spectrometer (Model 1600, Perkin-Elmer Corp., Norwalk, CT) with a diffuse reflection accessory. Samples were triturated with KBr (IR Grade, Fisher Scientific, Pittsburgh, PA) and spectra obtained with a 1-min run time (16 scans).

Nuclear Magnetic Resonance Spectroscopy—NMR analysis of the reaction products (10% w/v in chloroform-*d*, 1% v/v TMS) was

0022-3549/94/1200-1222$04.50/0

performed using a Bruker 250 Nuclear Magnetic Spectrometer (Bruker Instruments, Inc., Billerica, MA).

Organic Microanalysis—Samples were analyzed (Desert Analytics, Tucson, AZ) in duplicate for carbon, hydrogen, and oxygen; cholesteryl flufenamate was also analyzed for nitrogen.

Mass Spectroscopy—Mass spectra were obtained using direct insertion probe analysis (Model 5988A mass spectrometer, Hewlett-Packard Corp., Avondale, PA). The probe temperature was programmed from 80 to 325 °C at 30 °C/min. The instrument was scanned from *m/z* 50 to 700.

KCl/KBr Solution—A KCl/KBr solution with a density of 1.006 g/mL was prepared by adding 17.55 mL of saturated KBr solution (d = 1.3706 g/mL) to 1 L of 0.05 M KCl solution (d = 0.9996 g/mL).

Particle Size Determination—The particle size distribution of the microemulsion was determined using laser light scattering (Model N4MD submicron particle size analyzer, Coulter Electronics, Hialeah, FL) with a 90° detection angle at 25 °C. Samples were diluted with deionized water which had been filtered through a 0.2-μm Puradisc 25PP polypropylene filter (Whatman, Maidstone, England) to obtain a sample intensity between 1.0×10^5 and 2.0×10^5 counts/s. All determinations were run with a 1000 s run time.

Ultracentrifugation—Samples were centrifuged at 37 000 rpm (average relative centrifugal field = 90000g, range = 57000g to 122000g) for a h at 5 °C using an ultracentrifuge (L8-55 ultracentrifuge with a type 75Ti fixed angle rotor, Beckman Instruments, Inc., Palo Alto, CA).

HPLC Analysis—Microemulsions were assayed by HPLC using a modification of the method of Lize et al.[14] Analyses were run with a HPLC system consisting of a Series 400 solvent delivery system, LC-75 spectrophotometric detector (Perkin-Elmer Corp., Norwalk, CT), WISP 712 automatic sample injector (Waters Assoc., Milford, MA), and SP4270 integrating recorder (Spectra-Physics, San Jose, CA). Analyses were run using a mobile phase consisting of methanol:acetonitrile:tetrahydrofuran:water (290:400:400:120) with a flow rate of 1.0 mL/min on a Zorbax C-8 column, 4.6 mm i.d. × 15 cm (Dupont, Inc., Wilmington, DE). A 25-μL sample was injected onto the column, and peaks were detected at a wavelength of 214 nm. Retention volumes were 5.6 mL for DPPC, 10.2 mL for **3**, and 11.0 mL for **4**.

Eutectic Mixture Preparation—Binary mixtures were prepared of cholesteryl ibuprofen:cholesteryl oleate (CI:CO), cholesteryl ibuprofen:triolein (CI:TO), cholesteryl flufenamate:cholesteryl oleate (CF:CO), and cholesteryl flufenamate:triolein (CF:TO) in weight ratios of 10:90, 20:80, 30:70, 50:50, 70:30, and 90:10. To prepare the binary mixtures, solutions of the individual lipids were prepared in chloroform. Aliquots of the chloroform solutions were transferred to test tubes and combined to achieve the appropriate weight ratios for each of the compounds. After evaporation of the chloroform under a stream of nitrogen at 45 °C, the test tubes were then placed in a 45 °C vacuum oven for 24 h. Samples were then weighed into vented DSC pans and placed in a 3 °C refrigerator for a minimum of 24 h before testing.

Differential Scanning Calorimetry—DSC studies were conducted on 15-mg samples using a TA3000 system with a DSC-20 standard cell (Mettler Instruments Corp., Hightstown, NJ). In order to study transitions below room temperature, the DSC-20 standard cell was placed in a 1.5-cub ft refrigerator (Sanyo Corp., Tokyo, Japan) set at a temperature of 3 °C. All determinations were run in triplicate. Samples were scanned from 5 to 180 °C at 5 °C/min.

Solubility in Triolein—The solubilities of **3** and **4** were determined in triolein. A 1-g sample of triolein was weighed into a 4-mL screw-cap vial. After separate excess amounts of **3** and **4** were added to the vial, the vials were allowed to mix on a rotating mixer for 7 days. Samples were removed from each vial and filtered through a 0.8 μm-polycarbonate Nucleopore filter (Nucleopore Corp., Pleasanton, CA) to remove any undissolved material and weighed into a 5-mL volumetric flask. They were then dissolved in acetonitrile:tetrahydrofuran (50:50) and assayed for amount of cholesteryl ester by HPLC as described above.

Cholesteryl Ester Synthesis—All glassware was dried in an oven at 110 °C for 24 h prior to use. Reagents were weighed into three flasks. The flasks were evacuated, purged with argon 10 times, and sealed with a rubber septum. Addition of anhydrous methylene chloride and all transfers were performed using a gas-tight syringe. In a typical synthesis, cholesterol (3.70 g, 9.6 mmol) was weighed into a 50-mL pear-shaped flask and dissolved in 35 mL of anhydrous methylene chloride. Dicyclohexylcarbodiimide (1.86 g, 9.0 mmol) was weighed into a 25-mL pear-shaped flask and dissolved in 20 mL of anhydrous methylene chloride. A sample of **1** or **2** (1.79 g, and 1.86 g, respectively, 8.7 mmol) and 4-pyrrolidinopyridine (133 mg, 0.9 mmol) were weighed into a 250-mL two-neck round-bottom flask and dissolved in 10 mL of anhydrous methylene chloride.

During the reaction, a positive pressure of argon was maintained on the reaction flask by an argon-filled balloon on one neck of the flask; the other neck was sealed with a rubber septum. The cholesterol and the dicyclohexylcarbodiimide solutions were successively added to the reaction flask and stirred with a magnetic stirrer. The reaction was then allowed to stir at room temperature under argon overnight (typically 18–24 h).

Immediately after the addition of the dicyclohexylcarbodiimide solution, and periodically throughout the reaction, samples were removed with a syringe and spotted onto a 1 × 3 in. TLC plate. The reaction was considered complete when no evidence of the cholesterol or ibuprofen starting material was evident.

After allowing the reaction to stir overnight, a white precipitate formed which was identified as dicyclohexylurea by IR and GC–MS analysis. The reaction mixture was filtered (qualitative P4 filter paper, Fisher Scientific, Pittsburgh, PA) to remove the precipitated dicyclohexylurea and evaporated to dryness under vacuum at 45 °C. The resulting residue was dissolved in 10 mL of hexane and filtered a second time to remove any undissolved material.

Cholesteryl esters (**3** and **4**) were purified using column chromatography. The filtrate from above was applied to a 2.5 cm diameter × 7 cm length silica gel column and eluted with hexane. **3** and **4** were the first compounds to elute from the column as verified by TLC on 20 cm × 20 cm silica gel plates. Fractions containing **3** or **4** were combined, and the hexane was evaporated under vacuum at 45 °C.

For **3**, a typical yield was approximately 75%. **3** was recrystallized by dissolving in a minimum amount of hot 1-propanol (boiling point = 97.6 °C) and allowing to stand undisturbed until cooled to room temperature. The resulting product was a white amorphous solid which was then filtered under vacuum, washed with ice-cold 1-propanol, and dried in a vacuum oven at 40 °C overnight. A typical yield after recrystallization was approximately 50% overall.

For **4** a typical yield was approximately 63%. The resulting product was a white crystalline solid. A typical yield after recrystallization was approximately 60% overall.

In Vitro Cholesteryl Esterase Assay—Using the method of Heider and Boyett[15] the hydrolyses of cholesteryl oleate, cholesteryl palmitate, and cholesteryl benzoate were compared to those of **3** and **4**. Cholesteryl esters are hydrolyzed using a cholesteryl ester hydrolase (from porcine pancreas). The free cholesterol is oxidized by cholesterol oxidase, liberating H_2O_2. The H_2O_2 is coupled with p-hydroxyphenylacetic acid by the action of horseradish peroxidase to yield a chromogen which is detected fluorometrically at an excitation wavelength of 325 nm and an emission wavelength of 415 nm. Solutions of each of the cholesteryl esters were prepared in 2-propanol at a concentration equivalent to approximately 25 μg/mL of cholesterol. Total cholesterol reagent contained the following: sodium phosphate buffer, pH 7.0, 0.05 M; cholesteryl ester hydrolase, 0.08 IU/mL; cholesterol oxidase, 0.08 IU/mL; horseradish peroxidase, 30 IU/mL; sodium taurocholate, 5 mM; polyethylene glycol 8000, 0.17 mM; and p-hydroxyphenylacetic acid, 0.15 mM.

In triplicate, 40-μL aliquots of the cholesteryl ester solutions were placed in separate test tubes. To each tube was added 800 μL of the total cholesterol reagent, and the contents were mixed well. The tubes were incubated at 37 °C. After incubation periods of 1, 18, and 36 h, 1.6 mL of 0.5 N NaOH was added to each tube, and the contents were mixed well. Fluorescence was determined with an excitation wavelength of 325 nm and an emission wavelength of 415 nm (Model LS-5B fluorometric spectrometer, Perkin-Elmer Corp., Norwalk, CT).

Microemulsion Preparation—Dipalmitoylphosphatidylcholine (DPPC) and cholesteryl ester (CE) were dissolved in 1-propanol in a test tube and heated to 96 °C in a circulating water bath along with six test tubes (20 mm × 150 mm) containing a 10-mL aliquot of KCl/KBr solution, d = 1.006 g/mL. A 400-μL aliquot of the DPPC/CE solution was then slowly added dropwise to each of the six KCl/KBr solutions while being vortexed. Vortexing was continued for 5 min after the addition of the DPPC/CE solution. The size of the resulting translucent microemulsion was determined using the Coulter N4MD. For a typical microemulsion, with a molar ratio of DPPC:CI 80:20, DPPC (150 mg, 0.20 mmol) and **3** (30 mg, 0.05 mmol) were dissolved

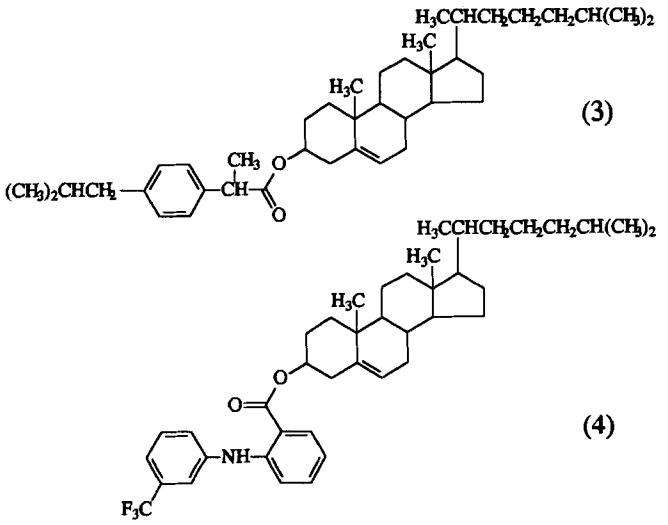


Figure 1—Structure of two cholesteryl ester prodrugs, cholesteryl ibuprofen (**3**) and cholesteryl flufenamate (**4**).

in 3 mL of 1-propanol, and 400-μL aliquots of the hot DPPC/CI solution were added to the hot KCl/KBr solution in each of six test tubes.

After the microemulsions cooled to room temperature, they were transferred to 10.4-mL screw cap ultracentrifuge tubes (Nalge Co., Rochester, NY) and centrifuged for 1 h at 37 000 rpm. The tubes were then carefully removed and the supernatant (fraction 1) was removed with a Pasteur pipet. This fraction contains material with a density of <1.006 g/mL. The pelleted material remaining in the tube was resuspended in 2.0 mL of KCl/KBr, d = 1.006 g/mL, by briefly ultrasonicating the centrifuge tubes in a water bath.

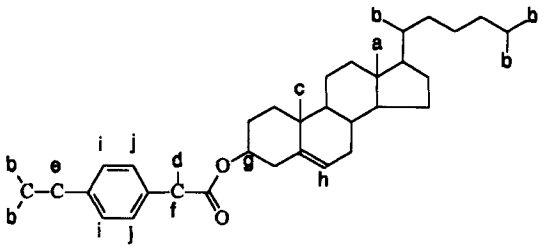
For the second ultracentrifugation, the density of the KCl/KBr solution was adjusted to d = 1.22 g/mL by adding 2.85 mL of saturated KBr solution to each tube. The microemulsions were then recentrifuged for 1 h at 37 000 rpm. The majority of the microemulsion floated to the top of the tube in a gel-like mass. This material was carefully removed with a Pasteur pipet and labeled fraction 2. Fraction 2, which contains material having a density between 1.006 and 1.22 g/mL, was used for all further studies. Pooled fractions from each of the six tubes were combined and resuspended in 0.9% sodium chloride to achieve a final volume of 10 mL. The material in the lower portion of the tube was removed along with any other material which had pelleted after the second ultracentrifugation and labeled Fraction 3. Fraction 3 contains any material with a density >1.22 g/mL.

III. Results

Using the method of Patel et al.,[12] two cholesteryl ester prodrugs, cholesteryl ibuprofen and cholesteryl flufenamate, have been synthesized. These two cholesteryl ester prodrugs, **3** and **4**, have not been previously reported in the literature. The proposed structures of **3** and **4** from the preceding synthesis are shown in Figure 1. Verification of the proposed structures was performed using infrared spectroscopy, proton nuclear magnetic spectroscopy, elemental analysis, and mass spectroscopy.

Cholesteryl Ibuprofen and Cholesteryl Flufenamate Characterization—Cholesteryl ibuprofen (3β-cholest-5-enyl [α-methyl-4-(2-methylpropyl)benzeneacetate) was isolated as a waxy white amorphous powder. As the ibuprofen starting material was a racemic mixture, one would expect **3** to be a racemic product. The optical purity of **3** was not determined. The melting range determined with a capillary melting point apparatus was 114–120 °C. This wide melting range could be due to the racemic nature of the product. The peak endotherm determined with DSC was 113.7 ± 0.1 °C, with a ΔH of 51.20 ± 2.14 J/g (n = 3).

Table 1—NMR Data for Cholesteryl Ibuprofen



Position	Chemical Shift (ppm)	Multiplicity	Integration
a	0.66	singlet	3H
b	0.85–0.92	doublet of doublets	15H
c	1.00	singlet	3H
d	1.45–1.48	doublet	3H
e	2.43–2.45	doublet	3H
f	3.60–3.72	quartet	1H
g	4.55–4.68	multiplet	1H
h	5.30–5.40	multiplet	1H
i	7.06–7.09	doublet	2H
j	7.18–7.21	doublet	2H

Cholesteryl flufenamate (3β-cholest-5-enyl 2-[[3-(trifluoromethyl)phenyl]amino]benzoate) was isolated as a white crystalline solid. The melting range determined with a capillary melting point apparatus was 145–148 °C. The peak endotherm determined using DSC was 147.3 ± 0.2 °C, with a ΔH of 56.20 ± 0.40 J/g (n = 3).

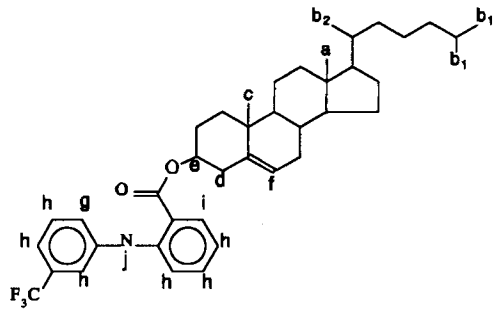
The infrared spectra of **3** and **4** were determined. The spectra show the presence of a strong sharp peak characteristic of the ester C=O stretch at 1731.5 cm^{-1} for **3** and 1676.0 cm^{-1} for **4**. The C=O stretch of **4** occurs at lower wavenumber characteristic of an ortho-amino ester. A group of moderately strong peaks, at 800.3–839.6 cm^{-1} for **3** and 792.9–831.1 cm^{-1} for **4**, are indicative of the ethylenic C–H bending of the Δ^5 bond of the cholesterol moiety.[16] In both spectra a strong group of peaks in the region 2800–3000 cm^{-1} due to the asymmetric and symmetric stretching vibrations of the aliphatic C–H bonds occur in the same region as for cholesterol. For **4** a strong sharp peak at 3307 cm^{-1} is characteristic of the N–H stretch of a secondary amine.[17] However in both spectra, the O–H bond stretching of the 3β-OH of cholesterol between 3220 and 3500 cm^{-1} and between 2350 and 3300 cm^{-1} due to the carboxylic OH of ibuprofen[16] is absent. These data are consistent with the formation of **3** and **4**.

The NMR spectra of **3** and **4** are summarized in Tables 1 and 2, respectively. The 3-αH signal of cholesteryl derivatives (at 3.8 ppm in cholesterol) is shifted downfield (at 4.5 ppm for **3** and 4.75 ppm for **4**), proving that the adjacent alcohol has been esterified. The NMR spectrum of **3** shows 10 major groups of peaks which can be attributed to the major peaks of both the ibuprofen and cholesterol moieties. The NMR spectrum of **4** shows 11 major groups of peaks which can be attributed to the flufenamic acid and cholesterol moieties.

Organic microanalysis was performed in duplicate. Determination of C, H, and O was performed for both. In addition, N was determined for **4**. The results are summarized in Table 3. The results of the elemental analysis are within 0.05% and 0.17% of the theoretical values for **3** and **4**, respectively, and further supports that the reaction products are cholesteryl ibuprofen, $C_{40}H_{62}O_2$, and cholesteryl flufenamate, $C_{41}H_{54}NO_2F_3$.

The mass spectra of **3** and **4** are summarized in Tables 4 and 5, respectively. Weak molecular ion peaks are evident at m/z = 574 for **3** and m/z = 649 for **4**. This is typical of most cholesteryl esters, as they will fragment into the cholesterol and acid moieties. For **3** a prominent peak at m/z

Table 2—NMR Data for Cholesteryl Flufenamate



Proton	Chemical Shift (ppm)	Multiplicity	Integration
a	0.69	singlet	3H
b1	0.85–0.86	doublet	6H
b2	0.87–0.88	doublet	3H
c	1.07	singlet	3H
d	2.46–2.49	doublet	2H
e	4.87–4.97	multiplet	1H
f	5.42–5.44	doublet	1H
g	6.82–6.85	triplet	1H
h	7.26–7.48	multiplet	6H
i	7.99–8.03	doublet	1H
j	9.69	singlet	1H

Table 3—Results of Organic Microanalysis of Cholesteryl Ibuprofen, $C_{40}H_{62}O_2$, and Cholesteryl Flufenamate, $C_{41}H_{54}NO_2F_3$

		% C	% H	% N	% O
3	Theoretical	83.56	10.87		5.57
	Determined	83.39	10.94		5.30
		83.58	10.95		5.85
	Avg	83.49	10.94		5.57
4	Theoretical	75.78	8.38	2.16	4.92
	Determined	76.11	8.27	2.02	4.63
		75.90	8.38	1.97	4.96
	Avg	76.00	8.32	1.99	4.79

Table 4—Mass Spectral Data for Cholesteryl Ibuprofen

Mass	Fragment	Relative Abundance (%)
574	M^+	1.0
368	CHOL – H_2O	100.0
353	368 – CH_3	18.8
260	368 – $C_6H_3CH_3$=CH	18.8
255	368 – 113	14.8
247	368 – $C_6H_3CH_3$=$CHCH_2$	18.5
213	255 – $CH(CH_2CH_2)$	13.2
161	IBU – CO_2H	59.7
83	113 – $2(CH_3)$	13.3
71	$CH_2CH_2CH(CH_3)_2$	15.4
57	$CH_2CH(CH_3)_2$	43.7

= 368 resulting from the cholesterol fragment and a strong peak at m/z = 161 resulting from the ibuprofen fragment give strong evidence that the reaction product is cholesteryl ibuprofen. For **4** a prominent peak at m/z = 369 is present resulting from the cholesterol fragment, suggesting a rearrangement has occurred, and a prominent peak at m/z = 281 resulting from the flufenamic acid fragment give strong evidence that the reaction product is cholesteryl flufenamate.

In Vitro Cholesteryl Esterase Hydrolysis—Table 6 shows the rates of in vitro enzymatic hydrolysis of **3** and **4** compared to those of cholesteryl oleate, cholesteryl palmitate, and cholesteryl benzoate. The rate of hydrolysis of the two cholesteryl ester prodrugs has been shown in vitro to be much

Table 5—Mass Spectral Data for Cholesteryl Flufenamate

Mass	Fragment	(%) Relative Abundance
649	M^+	5.6
369	CHOL – OH	29.7
353	CHOL – CH_3	3.3
281	280 + H^+	100.0
263	281 – H_2O	53.0
255	369 – 113 – H^+	5.1
247	369 – $C_6H_3(CH_3)$=CH	10.7
235	236 – H^+	8.1
216	235 – F	6.2
213	255 – $CHCH_2CH_2$	4.6
166	235 – CF_3	4.3
145	$C_6H_4CF_3$	20.4
83	113 – $2(CH_3)$	12.9
71	$CH_2CH_2CH(CH_3)_2$	10.6
69	CF_3	24.6
57	$CH_2CH(CH_3)_2$	19.1

Table 6—In Vitro Hydrolysis of Cholesteryl Esters with Porcine Pancreatic Cholesterol Esterase

	% Hydrolysis (Mean ± SD, n = 3)		
Cholesteryl Ester	1 h	18 h	36 h
Cholesteryl oleate	81.2 ± 5.7	100.0 ± 0.1	
Cholesteryl palmitate	100.0 ± 0.2		
Cholesteryl benzoate	87.2 ± 0.4	100.0 ± 0.2	
Cholesteryl ibuprofen	TLTQ[a]	23.4 ± 2.5	100.0 ± 0.1
Cholesteryl flufenamate	TLTQ[a]	25.7 ± 0.6	100.0 ± 0.2

[a] Too low to quantitate.

Table 7—Particle Size Distribution of Microemulsions as a Function of the Molar Ratio of Dipalmitoylphosphatidylcholine:Cholesteryl Ibuprofen

	% of Population[a] (Mean ± SD, n = 6)		
Molar Ratio DPPC:CI	<200 nm	200–1000 nm	>1000 nm
80:20	87.3 ± 12.3	11.8 ± 13.4	1.0 ± 2.4
75:25	94.2 ± 14.3	5.8 ± 14.3	0
70:30	87.2 ± 21.0	11.5 ± 21.7	1.3 ± 3.3
65:35	69.8 ± 19.1	24.5 ± 22.5	5.7 ± 13.9

[a] SDP intensity results are related to the number of particles in the size range

slower than the native cholesteryl ester, cholesteryl oleate, and cholesteryl palmitate. Complete hydrolysis of cholesteryl palmitate was observed with a 1-h incubation. Complete hydrolysis of cholesteryl oleate and cholesteryl benzoate occurred within 18 h. Complete hydrolysis of **3** and **4** was not observed until 36 h.

Lipid Composition Effects on Particle Size of the Microemulsion—Maximum cholesteryl ester prodrug loading into the microemulsion was investigated as a function of increasing particle size distribution. In Table 7, microemulsions of DPPC:CI were prepared as described above with DPPC:CI molar ratios from 80:20 to 65:35. The particle size distribution of the resulting microemulsions was determined and separated into three groups: <200, 200–1000, and >1000 nm. Microemulsions with a mean particle size of 100–150 nm were prepared. Microemulsions were prepared in groups of six (n = 6) and average results of the particle size distributions are shown. Since it was desired to obtain microemulsions with a size in the range of 100–150 nm, the population of interest was that with a mean particle size of <200 nm. To have a large percentage of particles in the range >200 nm was considered detrimental to the formulation.

The results summarized in Table 7 indicate that as the molar percent of dipalmitoylphosphatidylcholine is reduced to 65% there is a significant increase in the percentage of large

Table 8—Particle Size Distribution of Microemulsions as a Function of the Concentration of Total Lipids in 1-Propanol Solution during Preparation

Total Concentration of Lipids (mg/mL)	Percent of Population[a] (Mean ± SD, $n = 6$)		
	<200 nm	200–1000 nm	>1000 nm
30	86.2 ± 15.7	13.8 ± 15.7	0
60	87.3 ± 12.3	11.8 ± 13.4	1.0 ± 2.4
90	56.2 ± 12.8	19.7 ± 22.3	24.2 ± 21.8
120	31.7 ± 17.6	17.8 ± 22.5	50.3 ± 27.8

[a] SDP intensity results correlated to the number of particles in each size range.

particles (>200 nm) which are formed (one-way ANOVA, $p = 0.05$ level). There is no statistically significant difference in the percent of particles in the <200-nm range among the 80:20, 75:25, and 70:30 formulations (one-way ANOVA, $p = 0.05$ level). Although the 80:20 and 75:25 formulations were not statistically different, the microemulsions formed with a ratio of 75:25, on average, produced the lowest percent of large particles with only one of six trials producing particles in the range >200 nm. On the basis of these results the molar ratio of 75% dipalmitoylphosphatidylcholine to 25% cholesteryl ester was chosen for use in all future formulations.

Lipid Concentration Effects on the Particle Size of the Microemulsion—Using a molar ratio of 75% dipalmitoylphosphatidylcholine to 25% **3** a second experiment was conducted to determine what effect increasing the concentration of total lipids (DPPC + **3**) in the 1-propanol solution had on the particle size distribution of the microemulsions. In Table 8, as the concentration of lipid is varied from 30 to 120 mg/mL, the number of small particles formed falls off rapidly above 60 mg/mL. Solutions of DPPC:CI were prepared in 3.0 mL of 1-propanol with total lipid concentrations ranging from 30 to 120 mg/mL. Microemulsion preparation and particle size distribution determination were as described above.

The results indicate that, as the concentration of the lipids in the 1-propanol solution is increased above 60 mg/mL, the particle size of the resulting microemulsions is adversely shifted to a larger range. While the 30 and 60 mg/mL formulations were not statistically significantly different from one another, the 90 and 120 mg/mL formulations had a significantly greater number of large particles (>200 nm). Although there was on average a small percentage (1.0 ± 2.4%) of particles in the >1000 nm range with the 60 mg/mL formulations, it was felt that this would not adversely affect the future experiments since filtration through a 0.2-μm filter would remove these particles. On the basis of these results, a total lipid concentration of 60 mg/mL was chosen for use in all future formulations.

The aqueous phase of the microemulsions was assayed for the presence of the cholesteryl ester prodrug to determine if any of the cholesteryl ester prodrug had partitioned into the aqueous phase. Microemulsions were ultracentrifuged, and the aqueous phase was removed and assayed by HPLC for either **3** or **4** as previously described above. In all cases, there was no cholesteryl ester prodrug detected in the aqueous phase.

DSC Studies—DSC studies were conducted to evaluate if a low melting point eutectic mixture could be formed between the two cholesteryl ester prodrugs and cholesteryl oleate and to determine the reduction of their melting points with triolein. The solubilities of both cholesteryl ester prodrugs were determined in triolein.

Cholesteryl oleate eutectic mixtures with **3** and **4** are shown in Figures 2 and 3. Binary mixtures of CI:CO, CF:CO, CI:TO, and CF:TO were prepared. The peak DSC endotherms for pure cholesteryl ibuprofen and cholesteryl flufenamate were determined to be 119.7 ± 0.5 and 148.0 ± 0.4 °C,

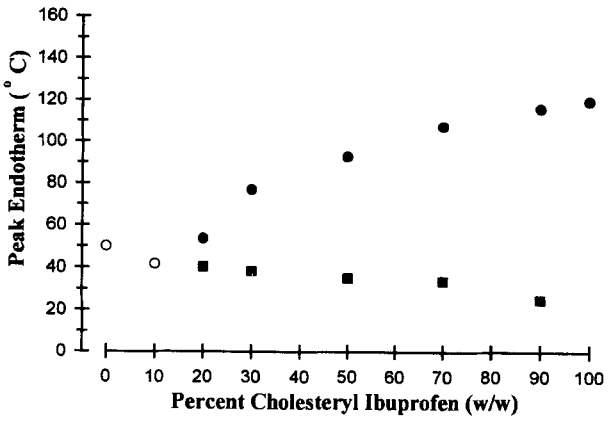


Figure 2—Phase diagram of binary mixtures of cholesteryl ibuprofen and cholesteryl oleate (Mettler TA3000 with DSC-20 standard cell, 5–180 °C at 5 °C/min: ● = cholesteryl ibuprofen, ○ = cholesteryl oleate, ■ = eutectic).

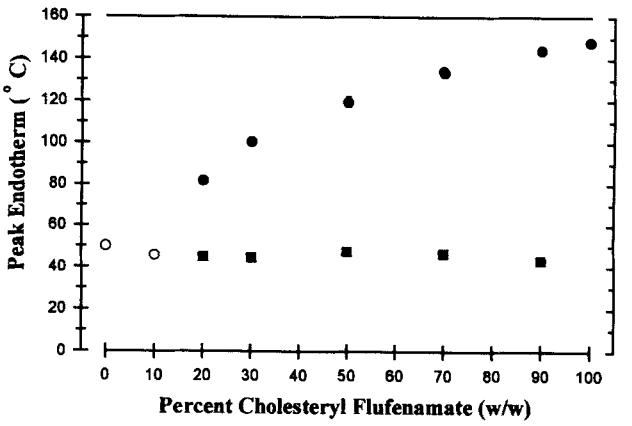


Figure 3—Phase diagram for binary mixtures of cholesteryl flufenamate and cholesteryl oleate (Mettler TA3000 with DSC-20 standard cell, 5–180 °C at 5 °C/min: ● = cholesteryl flufenamate, ○ = cholesteryl oleate, ■ = eutectic).

respectively. For the CI:CO diagram, extrapolation of the liquid–solid curve indicates a eutectic composition of approximately 16% cholesteryl ibuprofen and 84% cholesteryl oleate with a melting point of 35.2 ± 5.0 °C ($n = 14$). Similarly for **4**, the eutectic mixture composition was found to be approximately 12% cholesteryl flufenamate and 88% cholesteryl oleate with a melting point of 45.2 ± 1.7 °C ($n = 15$). The higher variability seen with the CI:CO mixtures may be due to the presence of a racemic product which results in a broader endotherm peak of **3**. With the broad peak it is more difficult to determine the peak endotherm temperature accurately. This is evident in Figure 3, where the eutectic melting point does not appear to be constant (±5.0 °C). Conversely, with **4** the endotherm peak is very sharp, allowing better accuracy in determining the peak temperature, resulting in a lower variability (±1.7 °C).

Binary mixtures of the two cholesteryl ester prodrugs with triolein also show a reduction of the melting point. The melting point of **3** was reduced from 119.7 ± 0.5 °C ($n = 3$) to 85.4 ± 2.0 °C ($n = 3$) at a composition of 30:70; similarly for **4**, a reduction from 147.8 ± 0.6 °C ($n = 3$) to 118.3 ± 2.0 °C ($n = 3$) was observed at a composition of 30:70. At lower concentrations of the prodrugs, complete solubility occurred. Since triolein has a melting point of 11 °C and exists as a liquid at physiological temperatures, the solubility of both cholesteryl ester prodrugs was determined in triolein. The solubilities of **3** and **4** in triolein were determined to be 13.2 ± 0.2% (w/w) and 11.5 ± 0.3% (w/w), respectively.

IV. Discussion

Several methods for the preparation of cholesteryl esters are available. The methods in the literature involve the reaction of cholesterol with acid halides,[12,18–20] reaction of cholesterol with acylimidazole derivatives of fatty acids,[21] reaction of cholesteryl chloride with acids,[22] and reaction of cholesterol with acid anhydrides.[23] The direct reaction of cholesterol with a carboxylic acid using the dehydrating reagent dicyclohexylcarbodiimide in the presence of an acylation catalyst[11,24] was used because of its simplicity and reported high yields. The method of Patel[12] gave cholesteryl esters of **1** and **2** in reasonable yields. No attempt was made to optimize the reactions. The incorporation of other cholesterol derivatives into lipoproteins and phospholipid microemulsions has been carried out in a number of cases. β-Sitosteryl β-D-glucopyranoside, a plant steroid, has been incorporated into lipoproteins to study its activity against P388 leukemia cells.[25] Deforge et al.[26] incorporated a radiolabeled cholesteryl iopanate into low-density lipoprotein (LDL) to study the disposition of LDL-associated cholesteryl esters in vivo. Similarly, other cholesterol derivatives including tris-[(galactosyloxy)methyl]aminomethane-terminated cholesterol, cholesteryl nitroxide, PCMA cholesteryl oleate, nitrobenzoxadiazole fluorophore derivative of cholesteryl linoleate, and sudan cholesteryl oleate have been incorporated into lipoprotein.[3] To date, however, there have been no reports of the incorporation of a cholesteryl ester prodrug into a phospholipid microemulsion for use as a drug-delivery system.

Since cholesteryl esters are hydrophobic they would be expected not to leach out of the core of the microemulsion. This is one advantage of microemulsions over vesicles, where a hydrophilic compound incorporated in the core of the vesicle may partition into the aqueous media. In addition, the cholesteryl ester prodrug may provide a means of slowly releasing the free drug in vivo. The in vitro hydrolysis of **3** and **4** was shown to occur at a much lower rate than the native cholesteryl esters cholesteryl palmitate and cholesteryl oleate. The substrate specificity of pancreatic cholesterol esterase has been studied. It has been shown that the binding of substrate to the cholesterol esterase is a hydrophobic interaction and is dependent on the chain length and degree of unsaturation.[27,28] The usual native substrates for cholesterol esterase are long chain hydrocarbon cholesteryl esters. The aromatic ring of **3** and **4** may have a steric effect preventing and reducing the interaction with the binding site of cholesterol esterase.

Two formulation variables were investigated to determine their effect on the particle size distribution of microemulsions: (1) the ratio of phospholipid:cholesteryl ester and (2) the total concentration of lipids (DPPC and CE) in the 1-propanol solution. On the basis of the results of this study, it was determined that a formulation with a DPPC:CE ratio of 75:25 resulted in a microemulsion with the least percentage of particles in the range >200 nm. Regarding total lipid concentration, preparation of the microemulsion with a 1-propanol concentration up to 60 mg/mL (DPPC + CE) resulted in microemulsions with the least percentage of larger particles. Increasing the concentration of the total lipids in the 1-propanol solution above 60 mg/mL resulted in the formation of a greater percentage of particles in the size range >200 nm. On the basis of these results, all microemulsions for in vivo studies were prepared from an alcoholic solution with a total lipid concentration of 60 mg/mL and a DPPC:CE molar ratio of 75:25.

Another strategy for increasing the concentration of the lipids in the microemulsion would have been to increase the volume of 1-propanol solution added to the KCl/KBr solution during the preparation. The addition of a larger volume of 1-propanol would have affected the density of the KCl/KBr solution. It was desired to keep the volume of 1-propanol added to the KCl/KBr solution to a minimum. In addition, increasing the percentage of 1-propanol may have changed the solubilities of the components in the KCl/KBr. Therefore, this strategy was not investigated.

Others in the literature have reported the formation of microemulsions with higher percentages of cholesteryl ester, but in all cases these investigators were able to prepare the microemulsions at a temperature above the melting point of all components.[5,6,29,30] Since both **3** and **4** have melting points above 100 °C, this is not possible. The KCl/KBr solution would boil off at the melting points of 120 and 148 °C, for **3** and **4**, respectively. The high melting point of the cholesteryl esters and their low aqueous solubility limit the ability to achieve microemulsions with a smaller mean particle size. Upon addition of the 1-propanol solution to the KCl/KBr solution, the cholesteryl esters would precipitate out of solution. This may result in crystals which cannot be reduced in size as opposed to a low melting cholesteryl ester which would remain fluid. This study demonstrates the utility and feasibility of incorporating high melting cholesteryl ester prodrugs into a phospholipid microemulsion.

Assay of the aqueous phase of the microemulsion after ultracentrifugation was performed. No **3** or **4** was detected in the aqueous supernate. This demonstrated that the hydrophobic cholesteryl ester prodrugs do not partition out of the microemulsion. These data demonstrate the benefit of forming a hydrophobic cholesteryl ester prodrug for incorporation into a phospholipid microemulsion. This strategy overcomes one of the disadvantages which is often reported for hydrophilic compounds formulated into vesicles with an aqueous core.

Other investigators have shown that the physical state of an emulsion particle substrate has an effect on the activity of cholesterol esterase in vitro.[31] In particular, they have demonstrated that if the core of a cholesteryl ester-containing emulsion exists in a liquid state the activity of the cholesterol esterase is grater. Eutectic mixtures of cholesteryl oleate with cholesteryl ibuprofen and cholesteryl flufenamate occurred at ratios of 84:16 (w/w%) and 88:12 with melting points of 35.2 ± 5.0 and 45.2 ± 1.7 °C, respectively. This raises the possibility that a formulation with CI:CO may exhibit a faster hydrolysis of the cholesteryl ester than one with CF:CO due to a eutectic with a melting point below the physiological temperature of 37.5 °C. Triolein formulations might also have faster hydrolysis rate since both **3** and **4** are soluble in triolein at 13.2% and 11.5% (w/w), respectively. With the CI:CO, CI:TO, and CF:TO formulations one would expect the core of the microemulsion to exist as a liquid at physiological temperature. Future studies will investigate the effect of the core composition on the elimination of the cholesteryl ester prodrugs in vivo.

Two cholesteryl ester prodrugs have been synthesized and characterized. The feasibility of formulating **3** and **4** into phospholipid microemulsions has been demonstrated. The microemulsions were optimized with respect to composition and resulting particle size. The microemulsions will be used in future studies to determine the feasibility of using a cholesteryl ester prodrug formulated into a phospholipid microemulsion for use as a drug-delivery system.

References and Notes

1. Lundberg, B. In *Lipoproteins as Carriers of Pharmacological Agents*; Shaw, J. M., Ed.; Marcel Dekker: New York, 1991; pp 97–139.
2. Fuchizawa, T. *J. Disp. Sci. Tech.* **1989**, *10*, 667–691.

3. Florence, A. T.; Halbert, G. W. In *Lipoproteins as Carriers of Pharmacological Agents*; Shaw, J. M., Ed.; Marcel Dekker: New York, 1991; pp 140–175.
4. Attwoood, D.; Florence, A. T. *Emulsions, Surfactant Systems, Their Chemistry, Pharmacy, and Biology*; Chapman and Hall: New York, NY, 1983.
5. Mims, M. P.; Guyton, J. R.; Morrisett, J. D. *Biochemistry* **1986**, *25*, 474–483.
6. Ginsburg, G. S.; Small, D. M.; Atkinson, D. *J. Biol. Chem.* **1982**, *257*, 8216–8227.
7. Nishikawa, O.; Yokoyama, S.; Kurasawa, T.; Yamamoto, A. *J. Biochem.* **1986**, *99*, 295–301.
8. Sklar, L. A.; Craig, L. F.; Pownall, H. J. *J. Biol. Chem.* **1981**, *256*, 4286–4292.
9. Via, D. P.; Craig, I. F.; Jacobs, G. W.; Van Vinkle, W. B.; Charlton, S. C.; Gotto, A. M.; Smith, L. C. *J. Lipid Res.* **1982**, *23*, 570–576.
10. Berthod, A.; Jin, H. L.; Beesley, T. E.; Duncan, J. D.; Armstrong, D. W. *J. Pharm. Biomed. Anal.* **1990**, *8*, 123–130.
11. Furniss, B. S.; Hannaford, A. J.; Smith, P. W. G.; Tatchell, A. R. *Vogel's Textbook of Practical Organic Chemistry*, 5th ed.; Longman Scientific and Technical Div., Wiley: New York, 1989; p 399.
12. Patel, K. M.; Sklar, L. A.; Currie, R.; Pownall, H. J.; Morrisett, J. D. *Lipids* **1979**, *14*, 816–818.
13. Gibson, H. W. *Mol. Cryst. Liq. Cryst.* **1974**, *27*, 43–53.
14. Lize, J. M.; Postaire, E.; Prognon, P.; Delattre, J.; Pradeau, D. *Pharm. Acta Helv.* **1988**, *63*, 239–240.
15. Heider, J. G.; Boyett, R. L. *J. Lipid Res.* **1978**, *19*, 514–518.
16. Gibbons, G. F.; Mitropoulos, K. A.; Myant, N. B. *Biochemistry of Cholesterol*; Elsevier Biomedical Press: New York, 1982.
17. El Hinnawi, M. A.; Najib, N. M. *Int. J. Pharm.* **1987**, *37*, 175–177.
18. Szabo, J. A.; Zoltai, A. I.; Motika, G.; Agocs, P. M.; Miklos, F. *Acta Phys. Chem.* **1979**, *25* (3–4), 167–71.
19. Helmich, O.; Hradec, J. *J. Labelled Compds. Radiopharm.* **1981**, *18* (5), 747–754.
20. Saroja, M.; Kaimal, T. N. B. *Synth. Commun.* **1986**, *16* (1), 1423–1430.
21. Grover, A. K.; Cushley, R. J. *J. Labelled Compd. Radiopharm.* **1979**, *16*, 307–313.
22. Heilbron, I. M.; Morton, R. A.; Sexton, W. A. *J. Chem. Soc.* **1928**, *1*, 47–51.
23. Lentz, B. R.; Barenholz, Y.; Thompson, T. E. *Chem. Phys. Lipids* **1975**, *15*, 216–221.
24. Dang, Q. Q.; Rogalle, P.; Puechmaurel, B.; Jollet, P.; Negre, A.; Vieu, C.; Salvayre, R.; Doust-Blazy, L. *Nato ASI Ser., Ser. A* **1988**, *159* (lipid disord.), 499–502.
25. Seki, J.; Okita, A.; Watanabe, M.; Nakagawa, T.; Honda, K.; Tatewaki, N.; Sugiyama, M. *J. Pharm. Sci.* **1985**, *74*, 1259–1264.
26. Deforge, L. A.; Degalan, M. R.; Ruyan, M. K.; Newton, R. S.; Counsell, R. E. *Nucl. Med. Biol.* **1992**, *19*, 775–782.
27. Kinnunen, P. M.; Klopf, F. H.; Bastiani, C. A.; Gelfman, C. M.; Lange, L. G. *Biochemistry* **1990**, *29*, 1648–1654.
28. Gandarias, J. M.; Lacort, M.; Martinez, M. J.; Fernandez, C. J.; Ochoa, B. *Steroids* **1987**, *49*, 403–417.
29. Craig, I. F.; Via, D. P.; Bette, C. S.; Sklar, L. A.; Mantulin, W. W.; Gotto, A. M.; Smith, L. C. *J. Biol. Chem.* **1982**, *257*, 330–335.
30. Parks, J. S.; Martin, J. A.; Johnson, F. L.; Rudel, L. L. *J. Biol. Chem.* **1985**, *260*, 3155–3163.
31. Lundberg, B. B.; Rothblat, G. H.; Glick, J. M.; Philips, M. C. *Biochim. Biophys. Acta* **1990**, *1042*, 301–309.

Acknowledgments

Financial support for this work was provided by the French/Gano/Kilmer/Pollard Predoctoral Fellowship of the Philadelphia College of Pharmacy and Science and by the Parke-Davis Pharmaceutical Research Division of the Warner-Lambert Co. Thanks to Jeffery Adams of Drexel University Chemistry Department, Philadelphia, PA, for running NMR analysis and National Medical Services, Willow Grove, PA, for performing mass spectral analysis.